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SPONSORED PROJECT INITIATION

Date: 11-17-80

Project Title: Active Site Studies on Blood Proteases

Project No: G-33-L03

Project Director: Dr. James C. Powers

Sponsor: DHEW/PHS/NIH - National Heart, Lung, & Blood Institute; Bethesda, MD 20014

Agreement Period: From 8/1/79 Until 7/31/81 (03 Year)

Type Agreement: Grant No. 5 R01 HL22530-03

Amount: $42,787 PHS Funds (G-33-L03)
3,314 GIT Contribution (G-33-333)
$46,101 TOTAL

Reports Required: Annual Progress Reports with Continuation Applications; Terminal Progress Report upon Grant Expiration

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Defense Priority Rating: None

Assigned to: Chemistry (School/Laboratory)

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SPONSORED PROJECT TERMINATION SHEET

Date 7/12/83

Project Title: Active Site Studies on Blood Proteases

Project No: G-33-L03

Project Director: Dr. James C. Powers

Sponsor: DHEW/PHS/NIH - National Heart, Lung, & Blood Institute; Bethesda, MD 20014

Effective Termination Date: 7/31/81

Clearance of Accounting Charges: 7/31/81

Grant/Contract Closeout Actions Remaining:

NONE

- ☐ **Final Invoice and Closing Documents**
- ☐ Final Fiscal Report
- ☐ Final Report of Inventions
- ☐ Govt. Property Inventory & Related Certificate
- ☐ Classified Material Certificate
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NOTE: Follow-on project (04 year) - G-33-L04

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studied enzymes such as Factor IX_a and XI_a. Thiol esters may also be useful as substrates for enzymes such as Factor XII_a where the complete sequence at the Factor XII_a cleavage site in Factor XI is not yet known. The second major aspect of our research involves synthesis and study of peptides with the sequences corresponding to plasma protease cleavage sites. We intend to measure their binding to various enzymes, their rates of cleavage and compare their reactivity with the reactivity of the natural substrates.

C. Progress Report. This project has been underway for 1 3/4 years starting on August 1, 1978.

Two predoctoral graduate research assistants have worked on the project. Brian McRae began work in April, 1979 on the synthesis of peptide thiol esters. Mario Castillo began work in February, 1979 on the synthesis of peptides with cleavage site sequences. Both have worked continuously on the project since that time. Full time graduate research assistants are listed at 40% time. I also received one month's salary (8.25% time) from the grant in the summers of 1979 and 1980.

The specific aims of the previous project were essentially identical with those of the current proposal and are stated above in Section A.

No publications have yet resulted from this research. One manuscript entitled "Mapping the Active Sites of Bovine Factor IX_a, Factor X_a, Factor XI_a, Thrombin and Trypsin with Amino Acid and Dipeptide Thiol Esters" by Brian J. McRae, K. Kurachi, Earl Davie and James C. Powers is in the stage of a first draft. I stopped work on that manuscript in order to complete this proposal before the June 1st deadline. I expect to submit the manuscript to Biochemistry in midsummer.

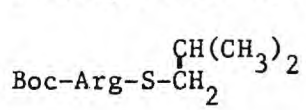
Amino Acid and Peptide Thiol Esters. One problem encountered with many of the synthetic substrates which have been studied with plasma serine proteases is the low sensitivity of the substrate which require the use of large amounts of enzyme for the assays. Amino acid and peptide thiol esters have been utilized recently in the development of sensitive assays for chymotrypsin (Farmer and Hageman, 1975), elastase (Castillo et. al., 1979), and trypsin (Green and Shaw, 1979). Cleavage of the thiol ester bond by the enzyme yields a thiol which is continuously determined spectrophotometrically by measuring the release of a chromogenic product upon reaction with a thiol reagent such as 2,2-dithiodipyridine contained in the assay mixture.

Thiol esters are good substrates for serine proteases and since thiol reagent can detect low levels of thiols, the substrates are quite sensitive. In a recent study of the merits of various chromophoric and fluorogenic leaving groups (Castillo et. al., 1979), we showed that an elastase assay using MeO-Suc-Ala-Ala-Pro-Val-$SCH_2C_6H_5$ was more sensitive than those using either the 4-nitroanilide or the aminomethylcoumarin leaving group. The above substrate could be used to detect human leukocyte elastase concentrations as low as 5 picomolar.

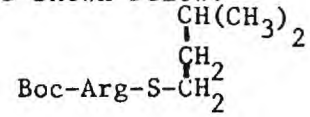
We have now shown that peptide thiol esters of the same type can be used in sensitive assays for the serine proteases of the coagulation and complement systems.

$$RCO\text{-}Arg\text{-}SR \rightarrow RCO\text{-}Arg\text{-}OH + RSH$$

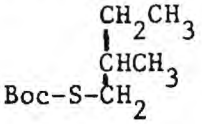
The synthesis of a series of amino acid and peptide thiol esters has now been complete and three of the simple derivatives are shown below.



Boc-Arg-SBu-i



Boc-Arg-SPe-i



Boc-Arg-SPe-s

The structures incorporate respectively the side chains of a P_1' valine, leucine and isoleucine residue. The activation sites of the zymogens of all known serine proteases involve cleavage of a Arg-Val, Arg-Leu, or Arg-Ile bond. These three substrates have allowed us to study the P_1' specificity of a number of plasma serine proteases.

The relative k_{cat}/K_M values for all the substrates are listed in the following Table. The substrates were studied at pH 7.5, 0.1 M HEPES buffer, 0.01 M $CaCl_2$, 98% v/v DMSO at 30° with trypsin, thrombin, Factor IX_a, Factor X_a and Factor XI_a.

Table [illegible] Relative k_{cat}/K_m values for the hydrolysis of the Synthetic Thiol Ester Substrates[a]

Substrate P_3 P_2 P_1	Enzyme: Trypsin	Thrombin	Factor IX_a	Factor X_a	Factor XI_a
Boc-Arg-SBu-i	70	50	0.0	1.0	4.7
Boc-Arg-SPe-i	139	63	0.0	0.87	2.1
Boc-Arg-SPe-s	50	80	0.0	0.73	2.8
Boc-Arg-SBzl	87	53	0.0	2.4	6.7
Z-Arg-SBu-i	220	31	0.0	2.6	5.7
Z-Gly-Arg-SBu-i	150	19	0.0	4.3	4.0
Z-Ala-Arg-SBu-i	93	23	0.0	2.0	3.2
Z-Val-Arg-SBu-i	130	3.3	0.0	0.67	2.0
Boc-Met-Arg-SBu-i	80	7.0	0.0	0.40	2.3
Z-Phe-Arg-SBu-i	220	29	0.37	1.3	4.0
Z-Trp-Arg-SBu-i	200	1.0	0.70	1.0	3.7
Boc-Pro-Arg-SBu-i	70	67	0.0	0.24	0.60
Z-Ser-Arg-SBu-i	160	4.7	0.0	1.1	3.7
Z-Thr-Arg-SBu-i	210	4.7	0.0	0.37	2.5
Z-Asn-Arg-SBu-i	150	5.7	0.0	1.0	5.7
Z-Glu-Arg-SBu-i	17	0.40	0.0	1.1	4.0
Z-Lys-Arg-SBu-i	83	0.93	0.0	0.43	3.3

[a] k_{cat}/K_m value for the hydrolysis of Boc-Arg-SBu-i by Bovine Factor X_a set at 1.0.

Values for all the kinetic constants (k_{cat}, K_M and k_{cat}/K_M) are listed in Tables in the appendix.

The kinetic parameters refer to the well established Scheme for serine proteases

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} E\cdot S \xrightarrow{k_2} E\cdot S' + P_1 \xrightarrow{k_3} E + P_2$$

where $E\cdot S'$ is the acyl enzyme. With esters and thiol esters $k_2 > k_3$ and k_{cat} which equals $k_2 k_3/(k_2+k_3)$ becomes the rate limiting deacytation rate (Hirohara et. al., 1977). The Michaelis constant $K_M = k_2 K_S/k_2$ and $k_{cat}/K_M = k_2/K_S$. Any non-productive binding modes would affect both k_{cat} and K_M, but would not change k_{cat}/K_M values.

The P_1' side chain appears to have little effect with any of the five enzymes studied, at least in the case of simple amino acid thiol ester derivatives. The k_{cat}/K_M values for the three thiol esters Boc-Arg-SBu-i, Boc-Arg-SPe-i and Boc-Arg-SPe-s with each of the five enzymes studied were quite similar.

We next decided to study dipeptide thiol ester with a variety of amino acid residues at the P_2 site in order to map the S_2 subsite of various trypsin-like enzymes. Eleven different amino acids with varing side characteristics are represented in the substrates listed in the table. Changing the P_2 residue caused the k_{cat}/K_M values to vary over a 168 fold range with thrombin, 18 fold with Factor X_a, and 10 fold with Factor IX_a. In each case a different P_2 residue was preferred: Pro for thrombin, Gly for Factor X_a; and Asn for Factor XI_a. In the case of thrombin and Factor X_a, these are the residues at the P_2 position of the natural substrates. The factor XI_a natural substrate has a P_2 Ser and the thiol ester with a P_2 Ser is just slightly poorer than the one with a P_2 Asn.

One highlight of our research to date is the discovery of two good synthetic substrates for Factor IX_a. Both the substrates (Z-Phe-Arg-SBu-i and Z-Trp-Arg-SBu-i) have a P_2 aromatic residue. As far as I know, there are no other good synthetic substrates for Factor IX_a. Interestingly, the kinetics with Factor IX_a are more complicated than with other enzymes. At present, it appears that the substrates are activating Factor IX_a at higher concentrations and we plan to study this phenomemon further.

We are also collaborating with Dr. T.Y. Lin (Merck, Rahway, New Jersey) in a study of the reactivity of the various thiol esters with the complement proteases $C1\bar{s}$ and $C1\bar{r}$. Only poorly hydrolyzed synthetic substrates are available for these enzymes (Andrews and Baillie, 1979). Although we have just begun the kinetic studies, it is evident that thiol esters are much more sensitive substrates for $C1\bar{s}$. The three single amino acid thiol esters listed earlier, have k_{cat}/K_M values with $C1\bar{s}$ in the range of $6.8 \times 10^3\ M^{-1}s^{-1}$.

The sensitivity of the various assays can be estimated from the k_{cat}/K_M values and our earlier experience with elastase (Castillo et. al., 1979). The best thrombin substrate should be useful for measuring enzyme concentrations in the range of 5 picomolar. The corresponding values with Factor X_a and XI_a would be 50 pM and with Factor IX_a, 500 pM. Unfortunately, the thiol esters are not specific enough to be used in situations where several of the coagulation factors are present.

In conclusion, we have developed a series of sensitive synthetic peptide thiol ester substrates for a number of plasma proteases, including enzymes (Factor IX_a, XI_a, $C1\bar{s}$) for which no other good synthetic substrates are available. These have allowed us to map the S_2 subsite of the various enzymes studied and are potentially useful in future studies of the active sites of various plasma proteases.

Fluorogenic Peptide Substrates. When we began this research, we planned to synthesize simple peptides corresponding to plasma protease cleavage sites and measure their hydrolysis rates using either a ninhydrin or fluorescamine method. Subsequently, we decided to change our strategy and to use fluorogenic peptides. A fairly new method for the assay of proteolytic enzymes involves the use of peptides containing both a fluoroscent and fluorescence-quenching group. Hydrolysis of the peptide with resulting

separation of the fluorescent and quenching groups results in an increase in fluorescence which can be used to follow the hydrolysis rate. This technique has been used in assays for carboxypeptidase (Latt et. al., 1972), trypsin (Carmel et. al., 1973), leucine aminopeptidase (Carmel et. al., 1977), and the angiotensin converting enzyme (Persson and Wilson, 1977).

We recently synthesized Abz-Ala-Gly-Leu-Ala-Nba (Abz = o-aminobenzoyl; Nba = 4-nitrobenzylamide) as a substrate for the Pseudomonas aeruginosa elastase (Nishino and Powers, 1980). The substrate has a small amount of intrinsic fluorecence, but complete hydrolysis led to a 6- to 8-fold increase in fluorescence (excitation, 340 nm; emission, 415 nm). The P. aeruginosa elastase splits the Gly-Leu bond, but cleavage at any of the peptide bonds would result in a fluorescence increase. The substrate has been used in our laboratory in a very sensitive rate assay for this elastase.

We decided to use this same technique in the study of peptides with sequences at plasma protease cleavage sites. Table VII in the appendix lists the 12 fluorogenic peptides we have synthesized to date. The sequences chosen initially correspond to Factor IX_a, XI_a and XII_a cleavage sites. Each peptide contains 5-7 amino acid residues encompassing residues on both sides of the peptide bond cleaved in the natural substrate. Since the sequence on the P side of the Factor XII_a cleavage site is not yet known, we simply placed three alanine residues at P_4-P_2 and synthesized Abz-Ala-Ala-Ala-Arg-Ile-Val-Gly-Nba to study with Factor XII_a.

The conformation of the individual peptides in solution determines to what extent the fluorescence of the o-aminobenzoyl group (Abz) is quenched and thus how much the fluorescence increases upon hydrolysis. We have found that long peptides are not necessarily quenched more poorly than the shorter ones. This indicates that many of the longer peptides have conformations in solution where the Abz and Nba groups are much closer to each other than if the peptides were in a fully extended conformation.

The hydrolysis of only two peptides have thus far been studied. Both were designed as Factor XI_a substrates. Abz-Lys-Leu-Thr-Arg-Val-Val-Gly-Nba is hydrolyzed by trypsin (K_M = 0.067 mM, k_{cat} - $17s^{-1}$, k_{cat}/K_M = 260,000 $M^{-1}s^{-1}$), Factor XI_a (K_M = 0.75 mM, k_{cat} =0.083 s^{-1}, k_{cat}/K_M = 111 $M^{-1}s^{-1}$) and Factor IX_a (k_{cat}/K_M = 18 $M^{-1}s^{-1}$), but not by Factor X_a or thrombin. Abz-Lys-Phe-Ser-Arg-Val-Val-Gly-Nba was hydrolyzed by trypsin (K_M = 0.083 mM, k_{cat} = $29s^{-1}$, k_{cat} = 360,000 $M^{-1}s^{-1}$) and Factor IX_a (k_{cat}/K_M = 73 $M^{-1}s^{-1}$), but not by Factor IX_a, X_a, or thrombin. The conditions were pH 7.5, 0.1 M HEPES, 0.01 M $CaCl_2$ at 30°C.

At this stage of the investigation only a few conclusions can be reached. First the rates of cleavage of the peptides by Factor XI_a are extremely slow compared to the trypsin cleavage rate. In each case we expect that Arg-Val bond is the one being cleaved, although we have no evidence for that at present. Importantly, the sequence appears to determine specificity. Neither peptide was cleaved after 48 hrs by thrombin or Factor X_a, while Factor IX_a cleaved the first peptide at only 16% of the rate observed with Factor XI_a.

The interaction of the other peptides listed in Table VII with Factors IX_a, X_a, XI_a, thrombin and trypsin will be studied in the next few weeks. The Factor XII_a substrate will be studied as soon as Factor XII_a is available.

D. Future Research Plans and Methods

Peptide Thiol Esters. We plan to carry out kinetic studies with any additional trypsin-like enzymes which become available to us. Specifically we will measure k_{cat}, K_M and k_{cat}/K_M values with the thiol ester substrates listed earlier in the table. The kinetic work with $\overline{Cls}$ should be finished shortly. We expect to obtain samples of Factor XII_a and kallikrein from Dr. Earl Davie in the near future. Since we have already synthesized the substrates, such kinetic studies consume relatively little time for the information that is obtained about the S_2 subsite of the various enzymes studied.

Additional synthetic work will be directed specifically toward Factor IX_a and Factor XI_a substrates, since these two enzymes have been poorly studied. In particular

we plan to synthesize Z-Phe-Ser-Arg-SBu-i, Z-Glu-Phe-Ser-Arg-SBu-i, and Z-Asp-Glu-Phe-Ser-Arg-SBu-i as Factor XI_a substrates and Z-Val-Val-Arg-SBu-i, Z-Gln-Val-Val-Arg-SBu-i and Z-Ser-Gln-Val-Val-Arg-SBu-i as Factor IX_a substrates. In each case we are extending the substrate by one amino acid residue at a time and are using the sequence at the cleavage site in the natural substrate. This will give us some idea as to the length of the extended substrate binding site in these two enzymes.

We also plan to synthesize the thiol esters Z-Ile-Ala-Gly-Arg-SBu-i and Z-Val-Ile-Ala-Gly-Arg-SBu-i. These peptides have respectively the P_4-P_1 and P_5-P_1 sequence at the reactive site of antithrombin III (Carrell et. al., 1980).[1] They might be expected to be equally reactive toward a number of coagulation serine proteases since antithrombin III inhibits most of these enzymes. We have found that peptides containing the sequence at the active site of the plasma inhibitor α_1-antitrypsin are hydrolyzed effectively by a number of serine proteases such as human leukocyte elastase and cathepsin G with quite different substrate specificities.

The substrate activation phenomenon observed with Factor IX_a will be studied further. In particular, we will first see if it is affected by added phospholipids such as a combination (1:1) of phosphatidylserine-phosphatidylcholine which is required for formation of a Factor VIII-Factor IX_a complex (Davie and Hanahan, 1977). It will also be interesting to see if phospholipids increase the Factor IX_a rate of thiol ester hydrolysis. We would also like to study the effect of Factor VIII on the rates, but it may be some time before enough pure Factor VIII is available for such studies.

One significant advantage of thiol ester substrates is their use of P' residues. Thus it is possible to design a substrate for an enzyme where only the N-terminal sequence released upon cleavage of its substrate is known. Thus a substrate such as Z-Arg-Sile-Val-Gly-Gly-NH_2 (Sile = $SCH(C_4H_9)CO$-) could be used to study or detect enzymes that activate Factor VII.

Our work with simple amino acid thiol esters described above indicates that the P_1' residue has little effect on hydrolysis rates. However, we would like to study a longer substrate before concluding that P' residues can be ignored in designing thiol ester substrates for plasma proteases. We have completed the synthesis of HSIle-Val-Gly-Gly-NH_2 and intend to couple it with Z-Arg-OH and study the resulting Z-Arg-Sile-Val-Gly-Gly-NH_2. This substrate has the P_1 through P_4' sequence at the Factor IX_a cleavage site and may be hydrolyzed by Factor IX_a. We will also study this peptide with the other enzymes which are available.

The thiol ester substrates which we have developed and are developing will be usful for many other investigators in their own studies. Dr. Roger Lundblad (U. of North Carolina) for example, has already requested one of our Factor IX_a substrates in order to study the binding of heparin to Factor IX_a.

Substrates with Other Leaving Groups. Once an appropriate thiol ester substrate for a protease is discovered, it would be useful to investigate corresponding derivatives where the thiol leaving group is replaced by aminomethylcoumarin (a fluorescent leaving group) or by 4-nitroaniline. In our study with elastase (Castillo et. al., 1979), we found the aminomethylcoumarins to be almost as sensitive as the thiol esters. The 4-nitroanilides in favorable cases were just slightly less sensitive and are very convenient to use. Both the aminomethylcoumarin and the 4-nitroanilide are amide substrates and could be used in situations where the thiol esters could not. For example, high concentrations of thiols or protein SH groups would interfer with the thiol ester assays, but not with the amide assays.

At present we are concentrating on Factor IX_a and XI_a substrates. Therefore it would appear that dipeptides such as Z-Phe-Arg-NA or Z-Phe-Arg-AMC (NA = 4-nitroanilide; AMC = aminomethylcoumarin) might be worth investigating as these correspond in sequence to one of our better dipeptide thiol ester substrates. However, it has been reported that Bz-Ser-Gln-Val-Val-Arg-NA is not cleaved by Factor IX_a (Suomela et. al., 1977). Therefore, we intend to do more mapping studies with longer thiol esters before looking at the corresponding nitroanilides or aminomethylcoumarins.

Fluorogenic Peptide Substrates. Our first priority is to carry out kinetic studies with the fluorogenic substrates which we already synthesized (listed in Table VII in appendix). These peptides have sequences corresponding to those found in Factor IX_a, Factor XI_a and Factor XII_a cleavage sites. The kinetic constants k_{cat} and K_M (equals K_S with peptides) will be determined.

If the peptides are not hydrolyzed by a particular enzyme, we will then measure its binding constant to that particular enzyme to see if it is binding, but not being cleaved. For these studies we will use our thiol ester substrates and treat the longer fluorogenic substrates as competive inhibitors. The K_I thus measured will give a measure of the binding of the peptides to the various enzymes. It is evident that our sensitive thiol ester substrates will allow us to study the binding of peptides to enzymes such as Factor IX_a, even if none of the simple peptides are hydrolyzed.

Studies with peptides should show us the length of the extended substrate binding region in proteases such as Factor IX_a and XI_a. As the peptide length is increased, a point should be reached where further interaction with the enzyme is not possible and no change in K_S (or K_I) occurs. For example, consider the 4 Factor IX_a substrates listed in Table VII. Each peptide is related to the previous one by having either one less or one more amino acid residue. Kinetic studies with this series of compounds will tell us the relative importance of extending substrates to encompass P_2, P_3, P_4 and P_3' residues.

Studies with peptides should also show just how important the conformation of the natural substrate is in determining the specificity of a plasma proteases. Specifically we will compare the rate of bond hydrolysis (k_{cat}) and the binding constant (K_M) obtained with the longer peptide substrates to the corresponding kinetic constants determined with the natural substrate. Many of the rate constants for cleavage of the natural substrates have appeared in the literature (Steinberg et. al., 1980). We would expect the K_M for the synthetic substrate to be higher than for the natural substrate and would conclude that the conformation of the substrate affected its binding to the protease. However, the situation is not so clearcut in the case of k_{cat}. One might expect k_{cat} to be similar in both cases, but it could be argued the conformation of the natural substrate might force the plasma protease into a more reactive conformation and increase k_{cat}. Experiments with synthetic peptides will help to answer such questions.

When we reach the point when we have obtained the best possible fluorogenic peptide substrate for each enzyme under study, it might be appropriate to synthesize a peptide with the same sequence, but lacking the Abz and Nba groups. This would show whether these two end groups have any effect on the hydrolysis rate.

The fluorescent peptides may be useful for study of the rates of binding of peptides to proteases. Undoubtly the conformation of a fluorogenic peptide will be altered upon binding to a particular enzyme. This should be reflected in a fluorescence change. For example, if a peptide such as Abz-Lys-Phe-Ser-Arg-Val-Val-Gly-Nba bound to Factor XI_a in a fully extended conformation, there should be almost no quenching of the fluorescence of Abz group due to the distance separating it from the nitrobenzyl quenching group. At low concentrations of substrate, we might be able to observe a fluorescence change simply upon binding to the enzyme this and could possibly allow us to measure a binding rate. At present, I don't know if our fluorescence spectrometer is sensitive enough to make such measurements.

Significance. The proposed research should lead to a better understanding of the nature of the active structures of various plasma proteases. We will obtain basic information concerning the interaction of these proteases with their substrates and natural inhibitors, and how their activity is affected by various plasma modulators such as phospholipids. The synthetic substrates which will be developed in this research should be quite sensitive and should allow assays to be developed for plasma components which are impossible currently due to the complex nature of clotting assays. This research may lead to new methods of disease diagnosis and could lead to new avenues of therapy where various specific activators or inhibitors

are used to control the activity of the proteases involved in important physiological processes such as blood coagulation, fibrinolysis and complement.

E. Facilities Available. The School of Chemistry at Georgia Institute of Technology moved into a new building in 1970. The facility contains ample research and office space. The department is well equipped in instrumentation relevant to the proposed research, including a high resolution mass spectrometer and several nuclear magnetic resonance spectrometers. A cold room is available in the building.

The laboratories of the principal investigator are well equipped for the synthesis of peptides and the measurement of their reaction with enzymes. The equipment includes a Thermovac freeze drying apparatus, pH staat, Beckman model 25 and model 35 uv-visible spectrometers, a fluorescence spectrometer and chromatographic equipment. We have constructed two high pressure liquid chromatography systems for the separation and purification of peptides. A stop flow spectrophotometer is available in the chemistry building if any rapid kinetic measurements are required.

F. Collaborative Arrangements. The coagulation proteases used in this research will be prepared and purified in the laboratory of Earl Davie at the Univ. of Wash. (see attached letter). The majority of the kinetic studies will be carried out at Georgia Tech, although some studies may be done at the U. of Wash. At present, we have ample supplies of bovine thrombin, Factor IX_a, Factor X_a and Factor XI_a. We expect to receive Factor XII_a and kallikrein shortly. $\bar{C}1s$ has been supplied to us by Dr. T. Y. Lin (Merck, Rahway, New Jersey). We also expect to study $\bar{C}1r$ when he completes the purification of that complement protease.

Principal Investigator Assurance

The undersigned agrees to accept responsibility for the scientific and technical conduct of the research project and for provision of required progress reports if a grant is awarded as the result of this application.

James C. Powers

James C. Powers
Principal Investigator